The differential display technique using short primers is not suited for the routine isolation of differentially expressed sequences in sugarcane

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A mRNA differential display technique using decamer primers was evaluated for the identification and isolation of differentially expressed gene sequences in the sugarcane culm. RNA was isolated from leafroll, leaf, mature culm and young culm tissues and reverse transcribed to cDNA. A series of 120 random decamer primers were used to amplify 1 767 fragments from the cDNA, resulting in an average of 15 fragments per primer. Thirty-five (2%) of these fragments were possible culm-specific sequences, and four of these were identified as putative culm-preferential rather than culm-specific fragments. None of these four fragments had significant sequence homology to sequences in the international databases. One of the fragments, SA11, was analysed using longer sequence-specific primers. Amplification products from cDNA and genomic DNA templates with these primers were of identical size. Results of a series of control reactions showed that the synthesis of the SA11 fragment from RNA was reverse transcription-dependent, and was not a product of genomic DNA contamination. Collectively the data indicate that this technique is not suitable for routine application in plants, especially those with complex genomes.

Keywords: cDNA, differential display, sugarcane, tissue-specific.

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Introduction

The differential display technique was developed to identify and characterise differentially expressed genes by detecting individual mRNA species and then isolating and analysing the cDNA (Liang & Pardee 1992; Liang *et al.* 1993). The method uses the polymerase chain reaction (PCR) to amplify even rare cDNAs, making the identification of differentially expressed genes of low abundance possible. It was claimed that the method could replace subtractive hybridisation techniques because it is less time consuming and allows the simultaneous handling of numerous samples. Comparison of RNA samples from different cells allows the identification and cloning of differentially expressed genes. A similar technique utilising a single randomly selected primer at low stringency for first and second strand cDNA synthesis, was also developed (Welsh *et al.* 1992).

Claimed advantages of the differential display technique are the low quantity of starting material required, simultaneous analysis of multiple samples and the speed at which the process can be completed (Miele *et al.* 1998). Drawbacks of the technique are the high incidence of false positives and the fact that the technique often shows a strong bias towards high copy number mRNAs (Bertioli *et al.* 1995). Clones derived from apparently single displayed bands frequently represent more than one different sequence, and the identification of the true differentially expressed fragments is thus hindered (Welsh *et al.* 1992; Callard *et al.* 1994; Sokolov & Prockop 1994; Hadman *et al.* 1995). Several modifications have been made to the original method and these include the preparation of DNA-free cytoplasmic RNA (Welsh *et al.* 1992; Bauer *et al.* 1993; Zimmerman & Schultz 1994; Zhao *et al.* 1995; Sompayrac *et al.* 1995; Luce & Burrows 1998), the use of different amplification primers (Liang *et al.* 1993; Sompayrac *et al.* 1995), and the use of different methods to display the amplified fragments (Sokolov & Prockop 1994; Lohmann *et al.* 1995, Doss 1996).

Internationally there has been an increased demand for the genetic manipulation of important crops including sugarcane (*Saccharum* spp.). These programmes are heavily dependent on the availability of promoter elements to target and control transgene expression. To date no specific sequences have been isolated from sugarcane. Our own research is focussed on the sugarcane culm as it is the main sucrose storing tissue and also the area being targeted for stalk borer control.

Secondary wall formation, suberin depositing (Jacobsen *et al.* 1992), and a large vacuole to cellular volume (Komor *et al.* 1981) make it very difficult to extract intact nucleic acid and especially mRNA from mature culm tissue. The apparent advantages of the differential display technique, therefore, appeared an appealing method for the isolation of culm-specific genes from sugarcane. The purpose of this was to use the differentially expressed sequences to isolate specific promoter elements.

Here we report that although numerous sequences were identified that appeared to be preferentially expressed, subsequent analysis failed to confirm this result. The general applicability of this technique for use in plants is discussed.

Materials and Methods

RNA Preparation

Total RNA was isolated from mature (6 to 12 month old) field-grown sugarcane plants (variety N19) using a modified method of Chomczynski and Sacchi (1987). The internode attached to the leaf with the uppermost visible dewlap was defined as internode 1. All solutions were prepared with water that had been treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC). Glassware was autoclaved and baked at 200°C prior to use, and plastic ware was used directly from sterile unopened packs. Tissue (5 g) was ground in liquid nitrogen and homogenised in 5 ml denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% (w/v) sarkosyl; 100 mM β-mercaptoethanol). After the addition of 0.5 ml of 2 M sodium acetate (pH 4.0), 5 ml phenol and 1 ml chloroform:isoamylalcohol (24:1), the suspension was shaken vigorously and left on ice for 15 min. After centrifugation at 10 000 g for 20 min at 4°C, the aqueous phase was removed and the RNA was precipitated for 1 h with an equal volume of isopropanol at -20°C. RNA was collected by centrifugation at 10 000 g for 20 min at 0°C. The pellet was dissolved in 1.5 ml denaturing solution and the precipitation was repeated. The resulting pellet was washed with 70% ethanol (room temperature) and dried under

vacuum. The RNA was resuspended in 250 µl DEPC-treated water at 65°C for 15 min. Isolated RNA was quantified spectrophotometrically and the integrity was verified by examining the 18S and 28S ribosomal fragments after separation in a formaldehyde-containing 1.5% (w/v) agarose gel. RNA preparations were stored at -80°C.

Reverse transcription

cDNA was synthesised in 20 µl volumes containing 1 µg total RNA, 20 pmol oligo(dT)$_{15}$ primer, 10 units placental ribonuclease inhibitor, 0.1 mM of each deoxynucleoside triphosphate (dNTP) and 4 units M-MuLV Reverse Transcriptase (Boehringer Mannheim). The reaction mixture was incubated at 37°C for 1 h.

Amplification of cDNA

Prior to amplification the cDNA was purified through a QIAquick-spin PCR purification column (QIAGEN). Single random decamer primers were used for PCR (Operon Technologies). The PCR reaction mixture contained 5 µl of cDNA template, 10 mM Tris-HCl, pH 8.3; 10 mM KCl; 3.8 mM $MgCl_2$; 0.2 µM random primer; 0.1 mM of each dNTP and 1 unit of Ampli*Taq* Stoffel fragment (Perkin Elmer) in a volume of 15 µl. The samples were overlaid with 30 µl mineral oil and subjected to 41 cycles of PCR using the following cycling parameters: 1 cycle of 94°C for 3 min, 35°C for 1 min and 72°C for 2 min with a 2.4°C s^{-1} ramp; 40 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 2 min using a 2.4°C s^{-1} ramp. The last cycle was followed by a 5 min extension at 72°C. PCR amplification products were separated in 2% (w/v) agarose gels in 1× TAE buffer (40 mM Tris-acetate; 1 mM EDTA, pH 8.0). After staining with ethidium bromide (0.01 mg ml^{-1}), the gels were photographed and the banding patterns analysed for the presence of tissue-specific fragments.

Isolation and re-amplification of possible tissue-specific fragments

Fragments that appeared to be unique to the culm tissue were punched out of the gel and placed in 100 µl TE buffer. Samples were incubated at 65°C for 10 min, vortexed and stored at 4°C. Five µl of this solution were used for re-amplification as described above. In cases where more than one re-amplification product was observed, the fragment of expected size was isolated from the gel using the QIAquick Gel Extraction Kit (QIAGEN). These isolated fragments were stored at -20°C.

Characterisation of isolated fragments by RT-PCR Southern blotting

Amplification products were transferred overnight from the agarose gels to nylon membranes (MagnaGraph, MSI) (Chomzcynski & Mackey 1994) and the DNA cross-linked to the nylon by ultraviolet light (2.5 min at 120 mJ cm^{-1}). Probes were prepared from specific isolated fragments using random primer labelling (Prime-It II; Stratagene) in the presence of [α-^{32}P]dCTP (Amersham; 3000 Ci mmol^{-1}). Prehybridisation and hybridisation were performed in a solution containing 5× SSC (150 mM NaCl; 15 mM tri-sodium citrate, pH 6.8), 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.5% (w/v) SDS, 5× Denhardt's solution [0.02% (w/v) of each bovine serum albumin (BSA), polyvinylpyrrolidone (PVP) and ficoll] and 100 µg ml^{-1} sheared herring sperm DNA. Prehybridisation of 4 h at 55°C was followed by overnight hybridisation at 55°C after the addition of the probe that was denatured by boiling for 5 min. The membrane was washed twice at room temperature for 20 min and once at 50°C for 20 min in a solution containing 2× SSC and 0.1% (w/v) SDS. This was followed by a 15 min wash at 50°C in 0.2× SSC and 0.1% (w/v) SDS. The membrane was exposed to X-ray film for 24 to 48 h.

Cloning of cDNA fragments

Fragments were cloned using the pCR-Script (SK+) cloning kit (Promega), but the *Epicurian coli* supercompetent cells were replaced with *E. coli* JM83 cells for transformation. Clones were identified using blue/white selection on X-Gal-containing LB-plates [1% (w/v) NaCl; 1% (w/v) tryptone; 0.5% (w/v) yeast extract; 1.5% (w/v) agar]. Insert sizes were determined by PCR amplification of the inserted fragments using the vector-specific T7 promoter and pUC18/M13 reverse primers.

Sequence determination

The *fmol*™ DNA sequencing system (Promega) was used together with the vector-specific T7 promoter primer and the M13/pUC18 reverse primer. A cycle sequencing protocol using direct incorporation of [α-^{35}S]dATP (Amersham) was followed, after which the sequencing products were separated in 6% (w/v) denaturing polyacrylamide gels. The gels were dried and exposed to X-ray film for 24 to 48 h. Sequences were read manually from the film.

Characterisation of the culm-preferential fragment, SA11, by specific amplification

Sequence specific amplification primers were designed to amplify the SA11 fragment. The primers were both 20-mers with a GC-content of 50–55%: SA11-F: 5'-ATC GCC GTC AAT GAG CAC TC-3' and SA11-R: 5'-AGA GGT TGT AGT GAC ATC GG-3'. Specific amplification reactions were done in 15 µl volumes using 1 µM of each primer. Different templates were used in these reactions and were as follows: cDNA from internode 7 tissue: 5 µl of purified reverse transcription mixture as described, RNA from leafroll and internode 7 tissues: 1 µg of total RNA, genomic DNA from sugarcane varieties NCo310 and 84F3097: 100 ng, and the cloned SA11 fragment as positive control: 15 ng. The rest of the PCR mixture was the same as described, except that it contained 2 units of *Taq* DNA Polymerase (Boehringer Mannheim) instead of the Ampli*Taq* Stoffel fragment. The cycling parameters were as follows: 1 cycle of 94°C for 2 min, 39 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 2 min. The last cycle was followed by a 7 min extension at 72°C. Amplification products were separated in 2% (w/v) agarose gels.

Characterisation of the culm-preferential fragment, SA11, by specific RT-PCR

The GeneAmp RNA PCR Kit (Perkin Elmer) was used for the specific RT-PCR of SA11. The reverse transcription reaction was done at 42°C for 15 min in a volume of 10 µl containing 5 mM $MgCl_2$, 1× PCR buffer II (supplied in the kit), 1 mM of each dNTP, 2.5 units of RNase inhibitor, 2.5 µM oligo(dT)$_{16}$ primer or 0.75 µM of the specific primer; 1 µg of total RNA and 5 units of M-MuLV Reverse Transcriptase, and was overlaid with 30 µl of mineral oil. The reaction was terminated by heating to 99°C for 5 minutes and then cooling to 5°C. The amplification reaction mixture was added directly to the cDNA after reverse transcription and contained 2 mM $MgCl_2$, 1× PCR buffer II, 2.5 units of AmpliTaq DNA Polymerase and 0.15 µM of each primer. This mixture was spun through the oil of the RT mixture and was subjected to 42 cycles of PCR using the following cycling parameters: 1 cycle of 94°C for 2 min, 40 cycles of 94°C for 30 s, 45°C for 45 s and 72°C for 3 min. The last cycle was followed by an 8 min extension at 72°C. The amplification products were separated in 2% (w/v) agarose gels.

Results and Discussion

The RNA isolation technique that was used yielded intact RNA averaging between 40 µg g^{-1} fresh mass (fm) tissue from the mature culm to 150 µg g^{-1} fm tissue from the leaf.

Instead of the sets of anchored dT primers that were used in the original method, a simple oligo(dT)$_{15}$ was used for reverse transcription to produce cDNA in a single reaction. Random hexanucleotides have also sometimes been used for reverse transcription to ensure that the amplified products were not all

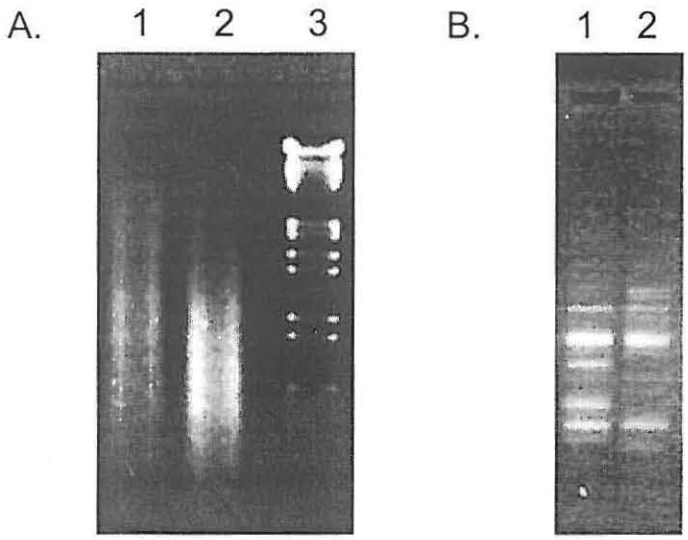

Figure 1 **A**. Amplification of cDNA using the oligo(dT)$_{15}$ and a random decamer primer. Reverse transcription was done using an oligo(dT)$_{15}$ primer after which 2.5 µl (lane 1) and 5.0 µl (lane 2) of the RT mixture was used in amplification reactions. Both the dT primer as well as a random decamer primer were used in this PCR. Molecular weight marker 3 is shown in lane 3. **B**. The oligo(dT)$_{15}$ primer was removed on completion of the reverse transcription reaction. Amplification was done using 2.5 µl (lane 1) and 5.0 µl (lane 2) of the undiluted purified cDNA, and a single random decamer primer.

situated on the 3' end of the mRNA (Sokolov & Prockop 1994). Initially, aliquots of 2.5 µl and 5.0 µl of the reverse transcription reaction mixture were used in amplification reactions and the oligo(dT)$_{15}$ primer was used together with a random decamer primer (Figure 1A). However, only smears were seen after the amplification reactions. Similar smears have also been observed by other groups (Liang *et al.* 1993; Hadman *et al.* 1995). The possibility that the template concentration was too high was investigated by using a dilution series of the reverse transcription reaction mixture ranging from 1:5 to 1:1 000 (results not shown). These dilutions also yielded smeared profiles.

Another possible explanation for these smears could be interference of the reverse transcription primer during amplification. The oligo(dT)$_{15}$ primer without anchoring 3' bases, could anneal to any part of the poly-A tail of a given mRNA. Subsequent reverse transcription will thus result in a series of amplified cDNA products of different sizes from the same mRNA.

Supporting evidence for this was obtained from the experiment where the oligo(dT)$_{15}$ primer was removed on completion of the reverse transcription reaction and amplification was done using a single random decamer primer. Undiluted purified cDNA was used in amplification reactions and a few discrete fragments were visible in the gel (Figure 1B).

Effect of template concentration

The effect of template (cDNA) concentration on the observed banding profiles of template was also investigated. A dilution series from mature culm (internode 7) cDNA ranging from 1:50 to 1:5 000 revealed that the only reproducible banding pattern was produced when dilutions of 1:50 and 1:100 were used (Figure 2). At lower template concentrations (higher dilutions) the banding patterns were highly variable between supposedly identical samples and, therefore, these template concentrations could not be used for differential display. In all subsequent reactions a 1:100 dilution of the purified reverse transcription reaction mixture, in which 1 µg of total RNA was reverse transcribed, was used as template. The concentration of the cDNA was not determined.

It is evident from these results that cDNA template concentration for RT-PCR is very important for the generation of reproducible amplification profiles and that furthermore, the differential display method is not a quantitative method.

Differential display: the comparison of random amplified fragments from different sugarcane tissues

A series of 120 random decamer primers was used in amplification reactions and cDNA fragments from leafroll, leaf, young culm (internode 2) and mature culm (internode 7) were compared. A total of 1 767 fragments were amplified, resulting in an average of 15 fragments per primer. The size of amplified fragments ranged from 100 to 2 000 bp. Thirty five (2%) of these fragments were identified as possible culm-specific fragments. Results obtained with some of the primers showed the presence of many polymorphisms, while with others the banding pattern was identical in all tissues.

To minimise false positives due to the synthesis of spurious fragments, two identical internode 7 samples were included in each reaction as suggested by Liang *et al.* (1993). This was not only used to verify banding profile reproducibility, but it also facilitated the characterisation of the isolated fragments.

Since this method relies on the amplification of cDNA, very low concentrations of contaminating DNA can skew the display of amplified expressed fragments. After each RNA isolation that was done, a control reaction was included in the first set of amplifications where the RNA was amplified without prior reverse transcription. This ensured that any genomic DNA contamination that might be present in the template would be detected. No amplification products were ever seen after these reactions (results not shown). Further confirmation that genomic DNA was not amplified during the differential display reactions was obtained by comparing the banding profiles to those obtained from random amplification of polymorphic DNA (RAPD) analysis of sugarcane genomic DNA using the same random primer. These profiles were very different (results not shown).

The average of 15 clear fragments per primer is comparable to the 10 to 20 amplified fragments that were detected by Welsh *et al.* (1992), and the 15 to 24 fragments that were amplified by Sokolov and Prockop (1994). The 2% of fragments that appeared to to be culm-specific is higher than the 1% differentially expressed fragments that were amplified by Liang *et al.* (1993).

Characterisation of isolated fragments using RT-PCR Southern blots

To determine whether the specific fragments that were isolated were highly related, as one would expect from sequences of isoenzymes or multi-gene families, the fragments were characterised by hybridisation analysis. Twenty one of the possible 35 culm-specific fragments were characterised using a RT-PCR Southern blot technique. These fragments were chosen because they could be successfully re-amplified or could be labelled directly without prior re-amplification. Fragments that did not re-amplify were not characterised further. Apparent unique fragments were identified, removed from the gel and used as a probe. A range of different results was obtained. Some fragments that were not tissue-specific hybridised strongly to the same sized fragments in all tissues, while others hybridised to fragments of different sizes in the same and other tissues indicating the possibility of the presence of isoforms or multi-gene families. In four cases the isolated fragment hybridised to the expected size

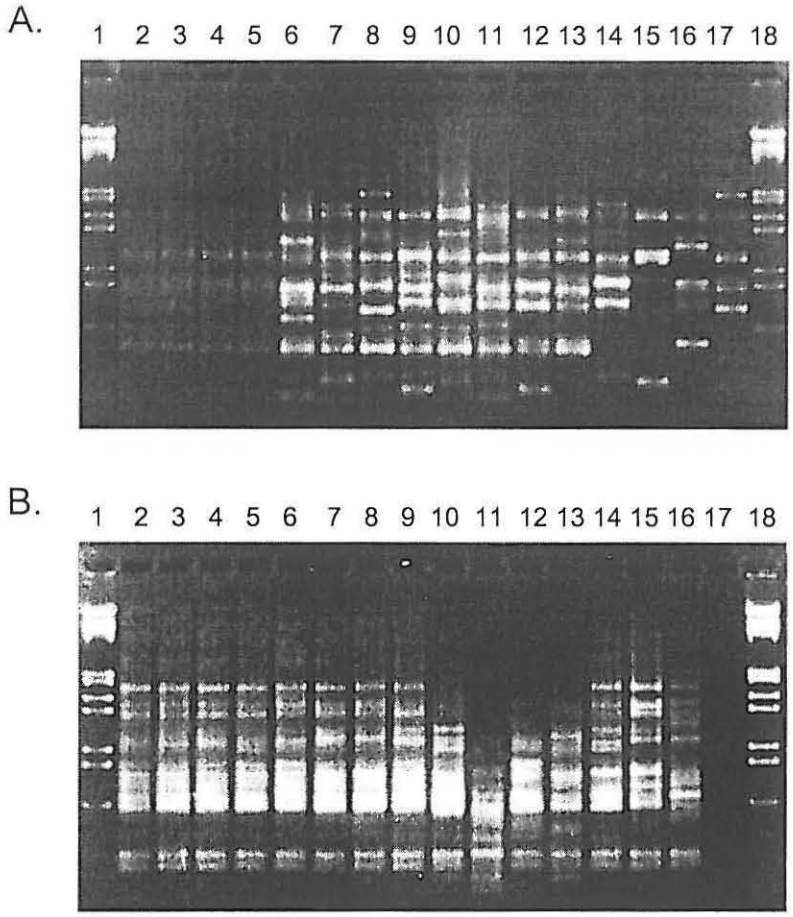

Figure 2 Optimisation of PCR template concentration for differential display. Dilution series of reverse transcribed cDNA from internode 7 were prepared. Four identical samples of each dilution were amplified to test the reproducibility of amplification profiles. **A.** Primer OPF12 was used in these reactions. Lanes 1 and 18: molecular weight marker, lanes 2–5: cDNA dilution 1:50, lanes 6–9: 1:500, lanes 10–13: 1:1000, lanes 14–17: 1:5000. **B.** Lanes 1 and 18: molecular weight marker, lanes 2–5: cDNA dilution 1:50, lanes 6–9: 1:100, lanes 10–13: 1:250, lanes 14–17: 1:500.

fragment in the culm and a weaker signal could be seen in other tissues. These fragments were termed culm-preferential. None of the characterised fragments were culm-specific.

The four culm-preferential fragments were designated 2A08 (667 bp), SA11 (640 bp), 7C16 (589 bp) and 7F18 (~1100 bp). The first part of the name identifies the tissue from which the fragments were isolated, i.e. internode 2, internode 7, or both of the culm tissues (S), while the second part indicates the primer

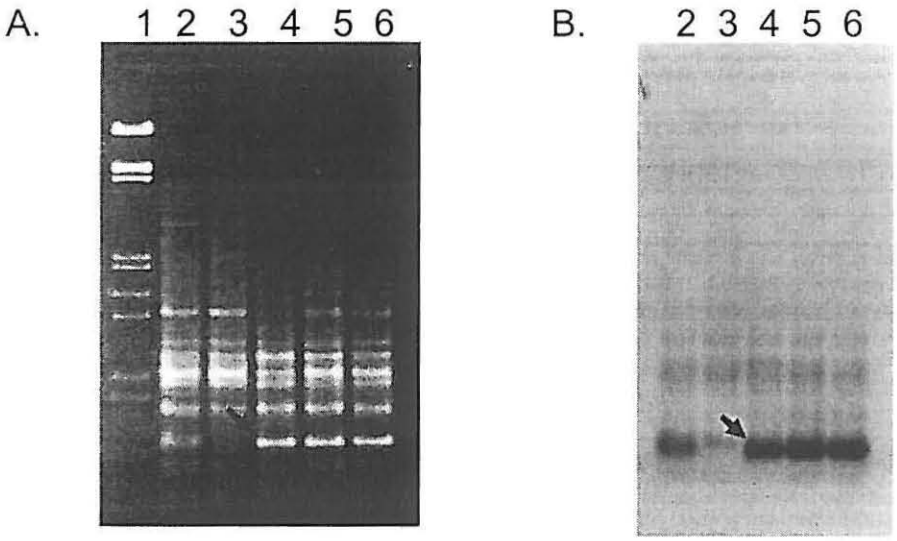

Figure 3 Identification and characterisation of culm-preferential fragment SA11. **A.** Amplification profile of sugarcane tissues produced by primer OPA11. The 640 bp fragment (SA11) visible in lanes 4, 5 and 6 is indicated with an arrow. **B.** Southern blot of the amplified fragments in (A) probed with isolated fragment SA11. The preferential hybridisation of the fragment to the same size fragments in the culm lanes (4, 5 and 6) is indicated with an arrow. Lane 1: molecular weight marker 3, lanes 2: leafroll, lanes 3: leaf, lanes 4: young culm (internode 2), lanes 5 and 6: mature culm (internode 7).

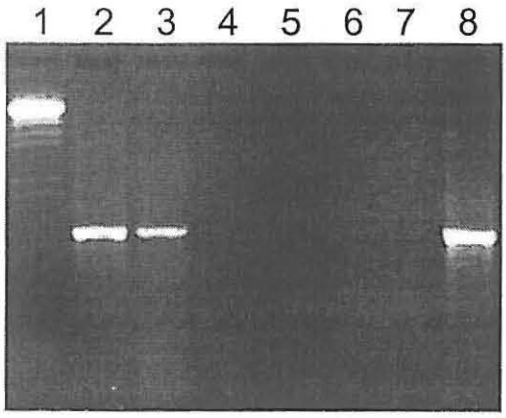

Figure 4 Specific amplification of fragment SA11 from cDN and genomic DNA. A concentration series of internode 7 cDNA wa prepared after the RT mixture was purified. Lane 1: molecula weight marker 3, lane 2: cDNA undiluted, lane 3: cDNA 1:10 dilu tion, lane 4: cDNA 1:50 dilution, lane 5: cDNA 1:100 dilution, lan 6: cDNA 1:250 dilution, lane 7: cDNA 1:500 dilution, lane 8: 120 n genomic DNA (variety N19).

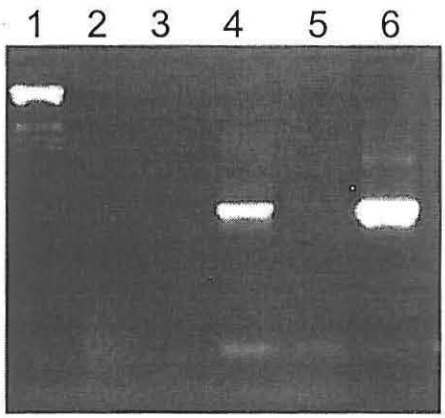

Figure 5 Determining the origin of fragment SA11. RNA from leafroll (lane 2) and internode 7 (lane 3) was amplified without prior reverse transcription. Genomic DNA from variety NCo310 (lane 4) and 84F3097 (lane 5) was amplified using the same primers. The cloned SA11 fragment was used as a positive amplification control (lane 6). Molecular weight marker 3 (lane 1) was used to determine the size of the amplified products.

that was used to amplify the fragment, e.g. OPA08. The differential display gel as well as the hybridisation result after RT-PCR Southern blot analysis of the culm-preferential fragment, SA11, is shown in Figure 3.

To verify that the isolated sequences truly represented mRNA in the original sample two approaches were followed. Firstly, the fragments were cloned and sequenced. Obtained sequences were compared to international databases and analysed for the presence of open reading frames. Secondly, sequence-specific long primers were developed for one of the fragments, SA11, for the amplification of the fragment from cDNA and genomic DNA. It was expected that amplification products from cDNA and genomic DNA would be of different lengths, because genomic fragments can contain both introns and exons.

Sequence determination of the cloned fragments

The complete sequences of 2A08, SA11 and 7C16 and a partial sequence of 7F18 were determined. The sequences were compared to both the non-redundant (NR) nucleotide sequence as well as the expressed sequence tag database (dbEST) by BLASTN, and to the deduced amino acid database by BLASTX (Altschul *et al.* 1990). No significant homologies were found, and only very short pieces of the fragments were aligned in the analyses by the basic local alignment search tool (BLAST) programmes. None of these fragments contained a continuous open reading frame.

Characterisation of the culm-preferential fragment, SA11

Sequence-specific 20-mer primers were designed for the amplification of this fragment. PCR using the 20-mer primers was done using cDNA from internode 7 and a genomic DNA template. The amplification products from both templates were of identical size (Figure 4 lanes 2 and 8).

The presence of genomic DNA contamination in the RNA samples was investigated by an amplification reaction where RNA from leafroll and internode 7 tissues, without prior reverse transcription, was used as template. No amplification products were formed from the RNA templates (Figure 5 lanes 2 and 3), but a 640 bp fragment was amplified from both the genomic DNA samples (Figure 5 lanes 4 and 5). In variety NCo310 (lane

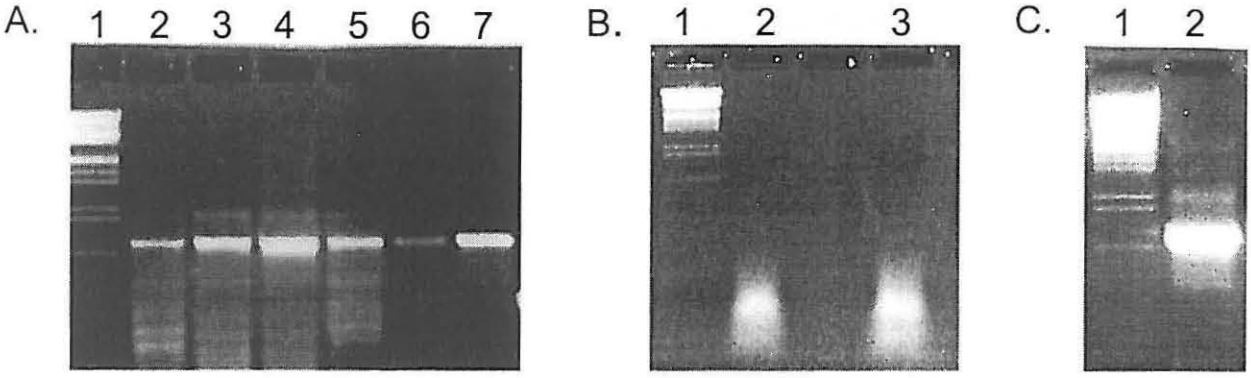

Figure 6 Dependence of the formation of fragment SA11 on reverse transcription. **A.** Reverse transcription of RNA from leafroll (lanes 2–4) and internode 7 (lanes 5–7) was primed with 3 different primers: SA11-F (lanes 2 and 5), SA11-R (lanes 3 and 6) and oligo$(dT)_{16}$ (lanes 4 and 7). Amplification of the reverse transcribed cDNA was done with SA11-F and SA11-R in all cases. **B.** Reverse transcription of RNA from leafroll using primers SA11-F (lane 2) and SA11-R (lane 3) without the addition of the reverse transcriptase enzyme. Subsequent amplification was done using SA11-F and SA11-R. **C.** Reverse transcription of RNA from leafroll using reverse transcriptase without the addition of a primer. Amplification was done using SA11-F and SA11-R (lane 2). In each case, the sizes of amplified fragments were determined with molecular weight marker 3 (lane 1).

4) a high concentration of the fragment was amplified, but in variety 84F3097 (lane 5), only a very faint product could be seen in the gel. In the positive control reaction (lane 6), the expected 640 bp fragment was amplified.

The fact that the isolated fragments did not contain continuous open reading frames may suggest that these fragments were derived from premature RNA transcripts as was previously reported (Luce & Burrows 1998). In that study at least one of five fragments that they had isolated was derived from an intron (Luce & Burrows 1998). In the present study the hypothesis that immature RNA transcripts could have been obtained was strengthened by the fact that during the acid guanidinium RNA isolation method that was used here nuclear membranes were disrupted. As a result of this procedure both nuclear and cytoplasmic RNA was isolated. Immature transcripts where splicing is not yet complete, but poly-A tail formation has already taken place will form part of the isolated RNA pool and can thus be amplified during the process of differential display.

The dependence of the synthesis of SA11 on the reverse transcription reaction was investigated by a series of RT-PCR reactions. Firstly, reverse transcription of RNA from leafroll and internode 7 tissues was primed with either the forward specific primer, SA11-F, the reverse specific primer, SA11-R, or the oligo$(dT)_{16}$ primer. Amplification of the reverse transcribed cDNA was done using the SA11-F and SA11-R primers, and the results are shown in Figure 6A. In all six reactions, the 640 bp SA11 fragment was synthesised. The only difference that was observed was that the fragments were amplified at different concentrations. Secondly, the RT-PCR was done using either SA11-R or SA11-F primers to prime reverse transcription, but no reverse transcriptase enzyme was added to the reaction. Amplification was then done using SA11-F and SA11-R primers and the results that were obtained are shown in Figure 6B. No amplification products were formed. In the third RT-PCR, no primers were used in the reverse transcription reaction, but after amplification with SA11-F and SA11-R, the 640 bp SA11 fragment had been synthesised as can be seen in Figure 6C.

Although we cannot fully explain the results obtained with SA11 the results question whether the amplified product is derived from poly-A mRNA. It is, however, evidently derived from single stranded RNA.

Conclusions

The differential display methods of Liang and Pardee (1992) and Welsh *et al* (1992) were combined and simplified to generate discrete cDNA bands from different sugarcane tissues with ease. In each step of the procedure the conditions had to be optimised for use with sugarcane.

Despite a relatively high incidence of apparent preferentially expressed sequences, none of the selected four fragments appeared to be coding sequences. These results indicate that caution is required in interpreting data that are obtained with this method.

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